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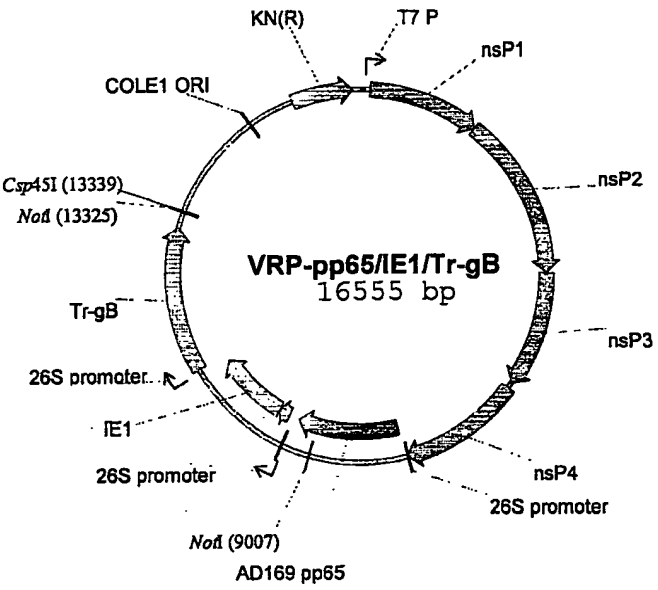

(57) Abstract: The present invention provides methods and compositions comprising a population of alphavirus replicon particles comprising alphavirus replicon RNAs, wherein a first replicon RNA comprises nucleic acid encoding cytomegalovirus pp65 and IE1 protein or immunogenic fragments thereof, and a second replicon RNA comprises nucleic acid encoding cytomegalovirus gB protein or an immunogenic fragment thereof, and wherein each of the two replicon RNAs is contained within a separate alphavirus replicon particle.

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ALPHAVIRUS-BASED CYTOMEGALOVIRUS VACCINES

RELATED APPLICATIONS

The present application claims the benefit, under 35 U.S.C. § 119(e), of U.S. provisional application serial number 60/486,501, filed July 11, 2003, the entire contents of which are incorporated by reference herein.

BACKGROUND OF THE INVENTION

Human cytomegalovirus (HCMV) is a herpesvirus that causes widespread infection found across all geographic locations and socio-economic groups, with up to 85% of adults infected by age 40 in the United States. For most healthy people who acquire the virus after birth there are no long-term consequences. However, the risk of HCMV infection is significant for several high-risk groups including: (i) unborn children, (ii) adults who work with children, and (iii) immuno-compromised persons. The prevalence of these risk groups underlies the importance of the development of a safe and efficacious vaccine.

HCMV is typically secreted via a number of bodily fluids, e.g., saliva, urine and semen. Thus, transmission of the virus between people can occur through either sexual or non-sexual contact. An individual can contract HCMV through blood or organ transplants, and a mother can transmit it to her unborn fetus.

The virus demonstrates a life-long latency, but is most commonly non-symptomatic in healthy individuals. It sometimes can cause an illness with symptoms similar to those associated with mononucleosis. However, it can cause severe illness in immunocompromised individuals, e.g., transplant recipients or those with acquired immunodeficiency syndrome (AIDS), in addition to the severe, debilitating effects on unborn children whose immune systems have not yet matured.

In the case of transplants, bone marrow transplant recipients show a relatively high incidence of HCMV-induced pneumonia, with consequent high mortality among these patients. In solid organ transplant patients, disease triggered by HCMV can include a HCMV syndrome (consisting of fever and leucopenia), hepatitis, colitis and pneumonia. HCMV-induced disease in these transplant recipients is caused by the immunosuppressive effects of

the drugs required for transplant acceptance and the induction of graft vs. host disease (GVHD). The GVHD effect is most severe in those instances where the organ/marrow donor is HCMV seropositive and the recipient is HCMV seronegative.

For AIDS patients, HCMV is the most common opportunistic infection, in large part due to the fact that greater than 90% of HIV-infected individuals are co-infected with HCMV. In these patients, the infection most commonly manifests as retinitis, and usually occurs when the CD4+ cell counts are less than 50/μl. Prior to the adoption of highly active antiretroviral (HAART) protocols, 20-44% of AIDS patients developed HCMV disease. While the use of HAART has also resulted in the reduction of HCMV disease, the unavailability of HAART for many AIDS patients, as well as the inability of many patients to tolerate HAART for extended periods of time, make the possibility of HCMV disease a continuing concern.

Congenital HCMV, a result of mother-to-fetus transmission, occurs at an overall rate of approximately 1%, but rates are much higher and symptomatic disease is more common when the mother has a primary infection. Women can be infected via sexual contact, since shedding of the virus from the cervix and in semen is common. Infected infants can remain viremic for up to five years after birth, becoming an important source for infection in day care settings.

Congenital HCMV can have horrific manifestations in infants. A fulminant cytomegalic inclusion disease can develop, characterized by jaundice, petechial rash, hepatosplenomegaly, microcephaly, and chorioretinitis. There is often progressive hearing loss and mental retardation, which can be severe. The estimated costs to society in terms of care for victims of congenital HCMV are approximately four billion dollars.

Thus, there remains a clear need for a safe and effective vaccine to combat HCMV infection, both prophylactically (for example, in adolescents or women of child-bearing potential to prevent congenital infection or in HCMV-uninfected transplant candidates) and therapeutically (for example, in HCMV-infected transplant patients prior to and after transplantation of an organ or bone marrow).

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a diagram of the replicon vector VRP-pp65/IE1/Tr-gB.

Figure 2 shows the results of a CMV neutralization assay following immunization of mice with various CMV-VRP vaccines.

Figures 3A-B show the results of an IFN-γ ELISPOT assay following immunization of mice with CMV-VRP vaccines. Figure 3A shows the results obtained by using peptide pp65 #3 and a pp65 peptide pool to evaluate cellular immune responses. Figure 3B shows the results obtained by using peptide IE1 #50 and an IE1 peptide pool to evaluate cellular immune responses.

SUMMARY OF THE INVENTION

The present invention provides a population of alphavirus replicon particles wherein said particles comprise alphavirus replicon RNAs, wherein a first replicon RNA comprises nucleic acid encoding cytomegalovirus pp65 and IE1 proteins or immunogenic fragments thereof, and a second replicon RNA comprises nucleic acid encoding cytomegalovirus gB protein or an immunogenic fragment thereof, and wherein each of the first and second replicon RNAs is contained within a separate alphavirus replicon particle.

Further provided herein is a population of alphavirus replicon particles wherein said particles comprise a replicon RNA which comprises a regulatory cassette that directs transcription and translation of a nucleic acid encoding cytomegalovirus pp65 and IE1 proteins, or immunogenic fragments thereof.

In additional embodiments, the present invention provides a population of alphavirus replicon particles wherein the particles comprise an alphavirus replicon RNA comprising nucleic acid encoding a CMV polypeptide selected from the group consisting of pp65, IE1, and gB, immunogenic fragments thereof or any combination thereof.

Also provided herein is a population of alphavirus replicon particles, wherein the particles comprise an alphavirus replicon RNA comprising nucleic acid encoding cytomegalovirus pp65 and gB proteins, or immunogenic fragments thereof.

The present invention also provides a population of alphavirus replicon particles comprising an alphavirus replicon RNA, wherein the replicon RNA of each particle comprises a first nucleic acid encoding cytomegalovirus pp65 protein or an immunogenic fragment thereof and a second nucleic acid encoding cytomegalovirus IE1 protein or an immunogenic fragment thereof, and wherein the expression of the first and second nucleic acid is controlled by separate regulatory cassettes.

Also provided herein are methods of inducing an immune response to CMV in a subject, comprising administering to the subject an effective amount of the populations of this invention.

Further provided is a method for inducing an immune response to CMV in a subject, comprising: a) priming the subject's immune system by administering to the subject an effective amount of a first immunizing component selected from the group consisting of: alphavirus replicon particles encoding CMV immunogens, CMV immunogens, nucleic acid molecules encoding CMV immunogens, a non-alphavirus viral vector encoding CMV immunogens, and any combination thereof; and b) boosting the subject's priming response by administering to the subject an effective amount of a second immunizing component selected from the group consisting of: alphavirus replicon particles encoding CMV immunogens, CMV immunogens, nucleic acid molecules encoding CMV immunogens, a non-alphavirus viral vector encoding CMV immunogens, and any combination thereof, wherein the first immunizing component is different from the second immunizing component and wherein at least the first immunizing component or the second immunizing component is an alphavirus replicon particle encoding CMV immunogens.

DETAILED DESCRIPTION OF THE INVENTION

As used herein, "a," "an," or "the" can mean one or more than one. For example, "a cell" can mean one cell or a plurality of cells.

"Alphavirus" means a genus of viruses, all of which are members of the Togaviridae family. Known alphaviruses include Eastern Equine Encephalitis virus (EEE), Venezuelan Equine Encephalitis virus (VEE), Everglades virus, Mucambo virus, Pixuna virus, Western Equine Encephalitis virus (WEE), Sindbis virus, South African arbovirus 86 (S.A.AR86) Semliki Forest virus, Middleburg virus, Chikungunya virus, O'nyong-nyong virus, Ross River virus, Barmah Forest virus, Getah virus, Sagiyama virus, Bebaru virus, Mayaro virus, Una virus, Aura virus, Whataroa virus, Babanki virus, Kyzylagach virus, Highlands J virus, Fort Morgan virus, Ndumu virus, and Buggy Creek virus. The alphaviral genome is a single-stranded, messenger-sense RNA, modified at the 5'-end with a methylated cap and at the 3'-end with a variable-length poly (A) tract. Structural subunits containing a single viral protein, capsid, associate with the RNA genome in an icosahedral nucleocapsid. In the virion, the capsid is surrounded by a lipid envelope covered with a regular array of transmembrane protein spikes, each of which consists of a heterodimeric complex of two glycoproteins, E1 and E2. See Pedersen et al., *J. Virol* 14:40 (1974). The Sindbis and Semliki Forest viruses are considered the prototypical alphaviruses and have been studied extensively. See Schlesinger, The Togaviridae and Flaviviridae, Plenum Publishing Corp., New York (1986). The preferred alphaviruses used in the constructs and methods of the claimed invention are VEE, S.AAR86, Sindbis (e.g., TR339, see U.S. Patent No. 6,008,035), and Semliki Forest Virus.

Within each named alphavirus, strains and/or subtypes are known. For example, several strains of the Venezuelan Equine Encephalitis virus (VEE) are known. Within the known strains of VEE, subtypes have been recognized. For example, the Trinidad Donkey strain is in subtype IA/B, and related subtypes include IC and IE. Virulent VEE strains have been isolated during mosquito-borne epizootic encephalomyelitis in equids in tropical and sub-tropical areas of the New World. The Trinidad Donkey strain is one of the virulent, epizootic strains, and it was passaged serially in tissue culture to create a live, attenuated strain (Berge et al. *Amer. J. Hyg.* 73:209-218 (1961)) known as TC-83. This strain, containing multiple attenuating mutations (see below, and Kinney et al. 1989 *Virology* 170:19-30 (1989); with correction noted in Kinney et al. *J Virol* 67(3):1269-1277 (1993)) elicits VEE-specific neutralizing antibodies in most humans and equines and has been used successfully as a vaccine in both species (e.g., Pittman et al. *Vaccine* 14(4):337-343 (1996)). Thus, the TC-83 strain of VEE can also serve as the genetic background for an alphavirus replicon vector system as described herein.

The terms "alphavirus RNA replicon," "alphavirus replicon RNA," "alphavirus replicon vector" and "alphavirus RNA vector replicon" are used interchangeably to refer to an RNA molecule expressing nonstructural protein genes such that it can direct its own replication (amplification) and comprises, at a minimum, the 5' and 3' alphavirus replication recognition sequences, coding sequences for alphavirus nonstructural proteins, and a polyadenosine tract. It may additionally contain a regulatory cassette and a heterologous nucleic acid of interest that is expressed from the regulatory cassette. It may also be engineered to express one but not all alphavirus structural proteins.

Specific embodiments of the alphavirus RNA replicons utilized in the claimed invention may contain one or more "attenuating mutations," an attenuating mutation being a nucleotide deletion, addition, or substitution of one or more nucleotide(s), or a mutation that comprises rearrangement or chimeric construction which results in a loss of virulence in a live virus containing the mutation as compared to the appropriate wild-type alphavirus. Several examples of attenuating mutations have been previously described in U.S. Patent Nos. 5,639,650, 5,792,462 and 6,156,558. Specific attenuating mutations for the VEE E1 glycoprotein can include an attenuating mutation at any one of E1 amino acid positions 81, 272 and/or 253. Alphavirus particles made from the VEE-3042 mutant contain an isoleucine substitution at E1-81, and virus particles made from the VEE-3040 mutant contain an attenuating mutation at E1-253. Specific attenuating mutations for the VEE E2 glycoprotein can include an attenuating mutation at any one of E2 amino acid positions 76, 120, and/or 209. Alphavirus particles made from the VEE-3014 mutant contain attenuating mutations at both E1-272 and at E2-209 (see U.S. Patent No. 5,792,492). A specific attenuating mutation for the VEE E3 glycoprotein includes an attenuating mutation consisting of a deletion of E3 amino acids 56-59. Virus particles made from the VEE-3526 mutant, now being developed as a vaccine strain, contain this deletion in E3 (aa56-59) as well as a second attenuating mutation at E1-253.

Specific attenuating mutations for the S.A.AR86 E2 glycoprotein include an attenuating mutation at any one of E2 amino acid positions 304, 314, 372, and/or 376 (see U.S. Patent No. 5,639,650). Alternatively, the attenuating mutation can be a substitution, deletion and/or insertion of an amino acid in the E2 glycoprotein, for example, at any one or more of the following amino acid positions in any combination: 158, 159, 160, 161 and/or 162

(see Polo et al., PCT Publication No. WO 00/61772, the entire contents of which are incorporated by reference herein).

Attenuating mutations can also be present in the alphavirus non-structural proteins, nsp1-nsp4. Exemplary attenuating mutations in the non-structural proteins for S.A.AR86 include, but are not limited to, codons which specify an attenuating amino acid at any one or more of the following: nsp1 amino acid position 538, nsp2 amino acid position 96, nsp2 amino acid position 372, nsp2 amino acid position 529; nsp2 amino acid position 571; nsp2 amino acid position 682; nsp2 amino acid position 804, nsp3 amino acid position 22, and in combination, codons at nsp2 amino acid positions 529, 571, 682 and 804 and at nsp3 amino acid position. Other illustrative attenuating mutations for S.A.AR86 include those described in PCT Application No. PCT/US01/27644.

Another type of attenuating mutation of this invention can be one or more attenuating mutations in the non-translated regions of the alphavirus genome which cause a loss in virulence in a live virus containing such mutations (e.g., see Niesters and Strauss "Defined mutations in the 5' non-translated sequence of Sindbis virus RNA" *J Virol* 64: 4162-4168 (1990)). One example of such a mutation is at nucleotide 3 of the VEE genomic RNA, *i.e.*, the third nucleotide following the 5' methylated cap (*see, e.g.*, U.S. Patent No. 5,643,576, describing a G→C mutation at nt 3; and White et al. "Role of alpha/beta interferon in Venezuelan Equine Encephalitis virus pathogenesis: effect of an attenuating mutation in the 5' untranslated region" *J Virol* 75:2706-2718 (2000)). The mutation can be a G→A, U or C, but the G→A mutation is preferred for some embodiments.

The term "alphavirus structural protein/protein(s)" refers to one or a combination of the structural proteins encoded by an alphavirus. These are produced by the virus as a polyprotein and are represented generally in the literature as C-E3-E2-6k-E1. E3 and 6k serve as membrane translocation/transport signals for the two glycoproteins, E2 and E1. Thus, use of the term E1 herein can refer to E1, E3-E1, 6k-E1, or E3-6k-E1, and use of the term E2 herein can refer to E2, PE2, E3-E2, 6k-E2, or E3-6k-E2. As discussed above for the replicon, specific embodiments of the alphavirus structural proteins utilized in the claimed invention may contain one or more attenuating mutations, an attenuating mutation being a nucleotide deletion, addition, and/or substitution of one or more nucleotide(s), or a mutation

that comprises rearrangement or chimeric construction which results in a loss of virulence in a live virus containing the mutation as compared to the appropriate wild-type alphavirus.

The terms "alphavirus replicon particles (ARPs)," "virus replicon particles," and "recombinant alphavirus particles," used interchangeably herein, mean a virion-like structural complex incorporating an alphavirus replicon RNA that expresses one or more heterologous RNA sequences. Typically, the virion-like structural complex includes one or more alphavirus structural proteins embedded in a lipid envelope enclosing a nucleocapsid that in turn encloses the RNA. The lipid envelope is typically derived from the plasma membrane of the cell in which the particles are produced. Preferably, the alphavirus replicon RNA is surrounded by a nucleocapsid structure comprised of the alphavirus capsid protein, and the alphavirus glycoproteins are embedded in the cell-derived lipid envelope. ARPs are infectious but propagation-defective, i.e., the replicon RNA cannot propagate beyond the host cell into which the particles initially infect, in the absence of the helper nucleic acid(s) encoding the alphavirus structural proteins. The structural proteins and replicon RNA of the ARPs may be derived from the same or different alphaviruses. In one embodiment, the replicon RNA and the structural proteins are both derived from VEE, and such particles are sometimes referred to herein as "VRP" or "VRPs". In another embodiment, the replicon RNA is derived from VEE and the structural proteins are derived from Sindbis virus (see, e.g., Dubensky et al., U.S. Patent No. 6,376,236).

The term "helper(s)" refers to one or more nucleic acid molecules capable of being expressed to produce one or more alphavirus structural proteins. The helpers can be RNA or DNA molecules. In one embodiment, the helper is a single DNA molecule comprising a promoter capable of directing the expression of nucleic acid encoding all the structural proteins of the alphavirus. In another embodiment, the helper comprises two RNA molecules that together express nucleic acid encoding all the alphavirus structural proteins. These two RNA molecules can be produced *in vitro*, or they can be generated from a single DNA helper that resolves itself into two separate molecules *in vivo*. In the case of the DNA helper constructs that do not employ alphaviral recognition signals for replication and transcription, the theoretical frequency of recombination is lower than the bipartite RNA helper systems that employ such signals.

The terms "helper cell" and "packaging cell" are used interchangeably herein and refer to the cell in which alphavirus replicon particles are produced. The helper cell comprises a set of helpers that encode one or more alphavirus structural proteins. As disclosed herein, the helpers may be RNA or DNA. The cell can be any cell that is alphavirus-permissive, i.e., cells that are capable of producing alphavirus particles upon introduction of a viral RNA transcript. Alphavirus-permissive cells include, but are not limited to, Vero, baby hamster kidney (BHK), 293, 293T, chicken embryo fibroblast (CEF), and Chinese hamster ovary (CHO) cells. In certain embodiments of the claimed invention, the helper or packaging cell may additionally include a heterologous RNA-dependent RNA polymerase and/or a sequence-specific protease.

The term "immunogenic fragment" means a fragment (e.g., a peptide) of a CMV protein that can stimulate either humoral or cellular immune responses in the host.

To stimulate the humoral arm of the immune system, i.e., the production of antigen-specific antibodies, an immunogenic fragment can include at least about 5-10 contiguous amino acid residues of the full-length molecule, preferably at least about 15-25 contiguous amino acid residues of the full-length molecule, and most preferably at least about 20-50 or more contiguous amino acid residues of the full-length molecule, that define an epitope, or any integer between five amino acids and the full-length sequence, provided that the fragment in question retains immunogenic activity, as measured by any art-known assay, such as the ones described herein.

Regions of a given polypeptide that include an epitope can be identified using any number of epitope mapping techniques, well known in the art. (See, e.g., Epitope Mapping Protocols in Methods in Molecular Biology, Vol. 66, Glenn E. Morris, Ed., 1996, Humana Press, Totowa, N.J.) For example, linear epitopes can be determined by e.g., concurrently synthesizing large numbers of peptides on solid supports, the peptides corresponding to portions of the protein molecule, and reacting the peptides with antibodies while the peptides are still attached to the supports. Such techniques are known in the art and described in, e.g., U.S. Pat. No. 4,708,871; Geysen et al. (1984) *Proc. Natl. Acad. Sci.* USA 81:3998-4002; Geysen et al. (1986) *Molec. Immunol.* 23:709-715, all incorporated herein by reference in their entireties.

Similarly, conformational epitopes are readily identified by determining spatial conformation of amino acids such as by, e.g., x-ray crystallography and 2-dimensional nuclear magnetic resonance. See, e.g., Epitope Mapping Protocols, supra. Antigenic regions of proteins can also be identified using standard antigenicity and hydropathy plots, such as those calculated using, e.g., the Omiga version 1.0 software program available from the Oxford Molecular Group. This computer program employs the Hopp/Woods method (Hopp et al., *Proc. Natl. Acad. Sci* USA (1981) 78:3824-3828) for determining antigenicity profiles and the Kyte-Doolittle technique (Kyte et al., *J. Mol. Biol.* (1982) 157:105-132) for hydropathy plots.

Generally, T-cell epitopes that are involved in stimulating the cellular arm of a subject's immune system are short peptides of about 8-25 amino acids, and these are not typically predicted by the above-described methods for identifying humoral epitopes. A common way to identify T-cell epitopes is to use overlapping synthetic peptides and analyze pools of these peptides, or the individual ones, that are recognized by T cells from animals that are immune to the antigen of interest, using, for example, an enzyme-linked immunospot assay (ELISPOT). These overlapping peptides can also be used in other assays such as the stimulation of cytokine release or secretion, or evaluated by constructing major histocompatibility (MHC) tetramers containing the peptide. Such immunogenic fragments can also be identified based on their ability to stimulate lymphocyte proliferation in response to stimulation by various fragments from the antigen of interest.

The term "epitope" as used herein refers to a sequence of at least about 3 to 5, preferably about 5 to 10 or 15, and not more than about 1,000 amino acids (or any integer therebetween), which define a sequence that by itself or as part of a larger sequence, binds to an antibody generated in response to such sequence or stimulates a cellular immune response. There is no critical upper limit to the length of the fragment, which can comprise nearly the full-length of the protein sequence, or even a fusion protein comprising two or more epitopes from a single or multiple CMV proteins. An epitope for use in the subject invention is not limited to a polypeptide having the exact sequence of the portion of the parent protein from which it is derived. Indeed, there are many known strains or isolates of CMV and the virus retains the ability to continue to adapt, and there are several variable domains in the virus that exhibit relatively high degrees of variability between isolates. Thus the term "epitope" encompasses sequences identical to the native sequence, as well as modifications to the

native sequence, such as deletions, additions and substitutions (generally, but not always, conservative in nature).

The term "regulatory cassette" means a nucleic acid sequence encoding one or more elements necessary to direct transcription and/or translation of a nucleic acid encoding one or more polypeptides. In one embodiment, the regulatory cassette comprises only an alphavirus subgenomic promoter to direct transcription of a cytoplasmically located RNA that is then capped, and the capped end of the RNA directs translation of the subgenomic RNA. In another embodiment, the regulatory cassette comprises an internal ribosome entry site, or IRES, which directs translation of a downstream coding region. In another embodiment, the regulatory cassette comprises an alphavirus subgenomic promoter and an IRES, and it is engineered to allow the subgenomic promoter to direct transcription (and thus amplification) of the RNA sequence downstream from the promoter and to allow the IRES to direct translation of the subgenomic RNA.

"IRES" means an internal ribosome entry site. IRES sequences have been found in numerous transcripts from viruses that infect vertebrate and invertebrate cells as well as in transcripts from vertebrate and invertebrate genes. Examples of IRES elements suitable for use in this invention include: viral IRES elements from Picornaviruses e.g., poliovirus (PV), encephalomyocarditis virus (EMCV), foot-and-mouth disease virus (FMDV), from Flaviviruses e.g. hepatitis C virus (HCV), from Pestiviruses e.g., classical swine fever virus (CSFV), from Retroviruses e.g., murine leukemia virus (MLV), from Lentiviruses e.g., simian immunodeficiency virus (SIV), or cellular mRNA IRES elements such as those from translation initiation factors e.g., eIF4G or DAP5, from Transcription factors e.g., c-Myc (Yang and Sarnow, *Nucleic Acids Research* 25:2800-2807 1997) or NF-κB-repressing factor (NRF), from growth factors e.g., vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF-2), platelet-derived growth factor B (PDGF B), from homeotic genes e.g., *Antennapedia,* from survival proteins e.g. X-Linked inhibitor of apoptosis (XIAP) or Apaf-1, or chaperones e.g. the immunoglobulin heavy-chain binding protein BiP (reviewed in Martínez-Salas et al., *Journal of General Virology* 82:973-984 (2001)).

Preferred IRES sequences that can be utilized in these embodiments are derived from: encephalomyocarditis virus (EMCV, ATCC accession # NC001479), cricket paralysis virus

(accession # AF218039), *Drosophila* C virus ATCC accession # AF014388, *Plautia stali* intestine virus (ATCC accession # AB006531), *Rhopalosiphum padi* virus (ATCC accession # AF022937), Himetobi P virus (ATCC accession # AB017037), acute bee paralysis virus (ATCC accession # AF150629), Black queen cell virus (ATCC accession # AF183905), Triatoma virus (ATCC accession # AF178440), *Acyrthosiphon pisum* virus (ATCC accession # AF024514), infectious flacherie virus (ATCC accession # AB000906), and Sacbrood virus (ATCC accession # AF092924). In addition to the naturally occurring IRES elements listed above, synthetic IRES sequences, designed to mimic the function of naturally occurring IRES sequences, can also be used. When more than one IRES is used in a replicon construct, the IRES elements may be the same or different.

"Boost" or "Booster" means a second immunization, after an initial (or "priming") immunization that enhances the immune response of the host. In one embodiment, the invention specifically provides a composition which produces an anamnestic response against a herpesvirus, e.g., CMV infection, in a sensitized subject, e.g., a horse, cow, or human, comprising an anamnestic response-inducing amount of a herpesvirus, e.g., CMV, immunizing component. As used herein, the term "anamnestic response" means a secondary (booster) immune response in a sensitized subject. By "sensitized subject" is meant a subject that has previously been in contact with herpesvirus, e.g., CMV, antigens either by natural exposure to the virus or by vaccination (primary immunization) with herpesvirus immunizing components, e.g., CMV –expressing alphavirus replicon particles.

At 230 kilobasepairs of double-stranded DNA, the cytomegalovirus genome is the largest β-herpesvirus known to infect humans. It has over 200 open reading frames responsible for encoding at least 165 genes; these are arranged in two segments, referred to as unique long (U_L) and unique short (U_S), which are separated by inverted repetitive nucleotide sequences. Thus, the choice of potential antigens to be used in a vaccine is quite large.

Some suggestions regarding vaccine approaches can be obtained from responses of healthy, seropositive individuals. In these individuals, 92% have CTLs present that target the pp65 antigen, 76% have CTLs to the IE1 antigen, 33% to the gB antigen, and 30% to the pp150 antigen (Gyulaj et al. 2000 *J. Infectious Diseases* 181:1537). In contrast, when this cell-mediated immunity is suppressed, the manifestations of HCMV disease are most severe. In addition, the reproductive number (number of cells infected by virus released from one

infected cell) is reduced 2 to 7 times in HCMV-experienced hosts. Replication of HCMV in HCMV-experienced immuno-compromised hosts is delayed compared to HCMV-naïve hosts (doubling time 0.38 days and 1.12 days, respectively). As a corollary, cellular responses to pp65 and IE1 have also been demonstrated to protect from CMV infection in animal models using the homologs of the HCMV genes encoding pp65 and IE1 (see Morello et al. *J. Virol.* 2000 Vol 74:3696). Finally, adoptive transfer of pp65-specific CTLs to bone-marrow transplant recipients protects them from CMV disease (Greenberg, P., Keystone Symposium April 2001; see also Walter et al., *N Engl J Med* 1995, 333:1038).

Antibodies to glycoprotein B (gB) are also present in HCMV infected individuals; these antibodies are neutralizing and have been implicated in protection of newborns from primary infection in animal models (Bourne et al. 2001 *J. Infectious Diseases* 183:59; Chatterjee et al. 2001 *J. Infectious Diseases* 183: 1547). Thus, it is expected that a preferred vaccine for humans provides both cellular and humoral immunity in order to be efficacious. Live, attenuated vaccines using the Towne strain of HCMV have been used in transplant patients to reduce the severity of transplant-induced CMV in those seronegative recipients who received a seropositive kidney (Plotkin 1994 *Transplantation* 58:1176). However, because of concerns regarding the safety of live, attenuated vaccines derived from a virus that causes a chronic, persistent infection that can be transmitted to the fetus, and that can be reactivated during periods of immunosuppression, alternative approaches are preferred.

The alphavirus replicon vector system provides the opportunity to induce robust humoral and cellular immunity in humans. The replicon vector system is based on the replication machinery of an alphavirus, consisting of a replicon RNA vector and one or more helper nucleic acids (reviewed in Rayner et al. (2002) *Rev. Med. Virol.* 12:279-96; see also U.S. Patent Nos. 5,792,462; 6,156,558; Pushko et al. (1997) *Virology* 239:389-401;U.S. Patent Publication No. 20020141975; PCT Publication No. WO 03/023026; the entire contents of which are incorporated herein by reference). The replicon RNA contains sequences required for replication and packaging of the RNA into a virus-like particle. It expresses the nonstructural proteins required for genome replication and transcription of subgenomic RNA (if such constructs are utilized), but lacks the structural protein genes necessary for formation of viral particles. The replicon is engineered so that a regulatory cassette can direct the expression of a nucleic acid of interest, in this invention, one ore more nucleic acids encoding CMV polypeptides or immunogenic fragments thereof. One or more

helper nucleic acids encode the alphavirus capsid and glycoproteins. When the replicon RNA vector and the one or more helper nucleic acids are introduced into an alphavirus-permissive cell, the replicon RNA is packaged into virus-like particles, which are harvested and purified to produce an immunogen, i.e., a vaccine composition.

In one embodiment, a replicon based on the Venezuelan Equine Encephalitis (VEE) virus is used as the vector for the CMV polypeptides. Nucleic acids encoding CMV proteins gB (e.g., from Towne strain), IE1 and pp65 (e.g., from AD169 strain) can be cloned into the alphavirus vector, e.g., the VEE vector, individually or in various combinations. Such combinations can encompass nucleic acid sequences encoding fusion proteins, e.g., IE1 and pp65 (or immunogenic fragments thereof) or they can encompass multiple regulatory cassettes within a single replicon, wherein each cassette drives either a single coding sequence or a nucleic acid sequence encoding a fusion protein of two or more CMV polypeptides, or immunogenic fragments thereof.

In one embodiment, each desired immunogen (i.e., a CMV polypeptide or an immunogenic fragment thereof) can be encoded by nucleic acid expressed in a separate alphavirus replicon particle, and mixtures of two or more ARPs, each comprising nucleic acid encoding a single CMV immunogen, can be prepared and administered to a subject. This invention therefore encompasses compositions comprising populations of one, two or three (or more) different ARPs. For example, in the case of three different ARPs, each ARP encodes a single CMV polypeptide, e.g., IE1, pp65, and gB (or immunogenic fragments thereof) and each ARP does not encode the other CMV polypeptides. In the case of two different ARPs, one ARP can express two CMV polypeptides, e.g., IE1 and pp65, either as fusion proteins or under the control of separate regulatory cassettes, as described above, and the second ARP can express one CMV polypeptide that is different, e.g., gB. Alternatively, a first ARP can express IE1 and a second ARP can express pp65. In another embodiment of a composition comprising two different ARPs, a first ARP can express pp65 and the second ARP can express gB. In an embodiment employing a single ARP, the replicon vector can include one regulatory cassette directing the expression of a nucleic acid encoding a fusion protein comprising at least pp65 and IE1, and in a specific embodiment, further comprising nucleic acid encoding gB. In another embodiment of the single ARP, one regulatory cassette can direct the expression of nucleic acid encoding gB, a second regulatory cassette can direct the expression of nucleic acid encoding IE1, and a third regulatory cassette can direct the

expression of nucleic acid encoding pp65. The regulatory cassette (RC)-encoding nucleic acid components can be in any order, e.g., RC-gB/RC-IE1/RC-pp65, and/or RC-pp65/RC-IE1/RC-gB. In another embodiment of the single ARP, a first regulatory cassette can direct the expression of a nucleic acid encoding a pp65-IE1 fusion protein, and a second RC can direct the expression of nucleic acid encoding gB. In all of the embodiments described herein, the use of the terms "pp65," "IE1," and "gB" are meant to encompass the full-length polypeptides, immunogenic fragments, and/or epitopes thereof. Nucleic acid sequences can be cloned from known CMV virus strains, e.g., Towne and AD169, and/or they can be synthetic sequences representing consensus sequences of the CMV sequences or epitopes from one or more CMV polypeptides, or chimeric sequences including fragments or epitopes from different CMV strains.

Thus, in certain embodiments, the present invention provides a population of alphavirus replicon particles wherein said particles comprise alphavirus replicon RNAs, wherein a first replicon RNA comprises nucleic acid encoding cytomegalovirus pp65 and IE1 proteins or immunogenic fragments thereof, and a second replicon RNA comprises nucleic acid encoding cytomegalovirus gB protein or an immunogenic fragment thereof, and wherein each of the first and second replicon RNAs is contained within a separate alphavirus replicon particle. In some embodiments, the first replicon RNA can direct transcription and translation of the nucleic acid encoding cytomegalovirus pp65 and IE1 proteins or immunogenic fragments thereof from two separate regulatory cassettes, whereby a first regulatory cassette directs transcription and translation of the CMV pp65-encoding nucleic acid and a second regulatory cassette directs replication and translation of the CMV IE1-encoding nucleic acid. In certain embodiments, the replicon RNAs of the particles of this population can further encode a CMV gB protein, and/or immunogenic fragment thereof and production of the CMV gB protein can be under the control of a separate, third regulatory cassette.

Further provided herein is a population of alphavirus replicon particles wherein the particles comprise a replicon RNA that comprises a regulatory cassette that directs transcription and translation of a nucleic acid encoding cytomegalovirus pp65 and IE1 proteins, or immunogenic fragments thereof.

Additionally provided herein is a population of alphavirus replicon particles wherein the particles comprise an alphavirus replicon RNA comprising nucleic acid encoding a CMV

polypeptide selected from the group consisting of pp65, IE1, and gB, immunogenic fragments thereof or any combination thereof.

In further embodiments, the present invention provides a population of alphavirus replicon particles, wherein the particles comprise an alphavirus replicon RNA comprising nucleic acid encoding-cytomegalovirus pp65 and gB proteins, or immunogenic fragments thereof. In some embodiments, this population can comprise alphavirus replicon RNA wherein the expression of the nucleic acid encoding cytomegalovirus pp65 and the expression of the nucleic acid encoding gB protein is controlled by separate regulatory cassettes.

A population of alphavirus replicon particles is also provided herein, comprising an alphavirus replicon RNA, wherein the replicon RNA of each particle comprises a first nucleic acid encoding cytomegalovirus pp65 protein or an immunogenic fragment thereof and a second nucleic acid encoding cytomegalovirus IE1 protein or an immunogenic fragment thereof, and wherein the expression of the first and second nucleic acid is controlled by separate regulatory cassettes.

In embodiments of this invention wherein the replicon RNAs direct expression of nucleic acid encoding CMV proteins and/or immunogenic fragments thereof of this invention from separate regulatory cassettes (e.g., one, two or three regulatory cassettes present on the same replicon RNA or on separate replicon RNAs of separate particles in a population), the regulatory cassettes can all be the same, the regulatory cassettes all be different and/or the regulatory cassettes can be present in any combination (e.g., two are the same and one is different).

In some embodiments, the regulatory cassette of this invention can be an alphavirus subgenomic promoter. In other embodiments, the regulatory cassette of this invention can comprise (i) an alphavirus subgenomic promoter to direct transcription, and (ii) an IRES element to direct translation.

In those embodiments of this invention wherein nucleic acid encoding CMV pp65 protein and nucleic acid encoding CMV IE1 protein are present on the same replicon, the nucleic acid can be present as a coding sequence that produces a fusion protein of pp65 and IE1. A nonlimiting example of a nucleic acid encoding a pp65/IE1 fusion protein is provided

as SEQ ID NO:3 and a nonlimiting example of an amino acid sequence of a pp65/IE1 fusion protein is provided herein as SEQ ID NO:4. Other nucleic acids encoding the pp65/IE1 fusion protein of this invention would be readily determined by one of ordinary skill in the art and would vary based on the degeneracy of the DNA code. Other amino acid sequences having the functional characteristics of the pp65/IE1 fusion protein of this invention would be readily determined by one of ordinary skill in the art and would vary based on, for example conservative amino acid substitutions, as well as deletions and/or additions having a neutral or nominal effect on the functional characteristics of the fusion protein.

In those embodiments of this invention wherein the replicon RNA comprises nucleic acid encoding CMV gB protein or an immunogenic fragment thereof, the transmembrane domain of the gB protein or an immunogenic fragment thereof can be present or it can be deleted. A nonlimiting example of a nucleic acid encoding a CMV gB protein that has been truncated to delete the transmembrane domain is provided herein as SEQ ID NO:1. A nonlimiting example of an amino acid sequence of a truncated CMV gB protein is provided herein as SEQ ID NO:2. Other nucleic acids encoding the gB protein of this invention would be readily determined by one of ordinary skill in the art and would vary based on the degeneracy of the DNA code. Other amino acid sequences having the functional characteristics of the gB protein of this invention would be readily determined by one of ordinary skill in the art and would vary based on, for example conservative amino acid substitutions, as well as deletions and/or additions having a neutral or nominal effect on the functional characteristics of the gB protein.

Immunogenic fragments of the CMV proteins of this invention would be readily identified by one of ordinary skill in the art according to standard methods for identifying regions of immunogenicity in an amino acid sequence. Nonlimiting examples of immunogenic fragments of this invention are provided in the Sequence Listing included herewith and identified as SEQ ID NOs:5-262. These immunogenic fragments can be employed in any combination and in any ratio relative to one another in the compositions and methods of this invention. For example, "pools" of peptides can be created according to protocols standard in the art (see, e.g., Maecker et al. "Use of overlapping peptide mixtures as antigens for cytokine flow cytometry" *Journal of Immunological Methods* 255:27-40 (2001)) and used to evaluate the immune response in subjects infected with HCMV or immunized with HCMV vaccines to identify immunogenic fragments.

The CMV-expressing ARPs of this invention are formulated for use as pharmaceutical formulations, vaccines or immunogenic compositions, either for prophylaxis and/or treatment. These pharmaceutical formulations comprise a composition of this invention (e.g., infectious, propagation-defective ARPs) in combination with a pharmaceutically acceptable carrier.

Thus, in certain embodiments, the present invention provides a composition comprising an alphavirus particle of this invention in a pharmaceutically acceptable carrier. The compositions described herein can be formulated for administration in a pharmaceutical carrier in accordance with known techniques. *See, e.g.,* Remington, *The Science And Practice of Pharmacy* (latest edition). In the manufacture of a pharmaceutical composition according to embodiments of the present invention, the composition of this invention is typically admixed with, *inter alia*, a pharmaceutically acceptable carrier. By "pharmaceutically acceptable carrier" is meant a carrier that is compatible with other ingredients in the pharmaceutical composition and that is not harmful or deleterious to the subject. The carrier may be a solid or a liquid, or both, and is preferably formulated with the composition of this invention as a unit-dose formulation. The pharmaceutical compositions are prepared by any of the well-known techniques of pharmacy including, but not limited to, admixing the components, optionally including one or more accessory ingredients. Exemplary pharmaceutically acceptable carriers include, but are not limited to, sterile pyrogen-free water and sterile pyrogen-free physiological saline solution. Such carriers can further include protein (e.g., serum albumin) and sugar (sucrose, sorbitol, glucose, etc.)

The pharmaceutical compositions of this invention include those suitable for oral, rectal, topical, inhalation (e.g., via an aerosol) buccal (e.g., sub-lingual), vaginal, parenteral (e.g., subcutaneous, intramuscular, intradermal, intraarticular, intrapleural, intraperitoneal, intracerebral, intraarterial, or intravenous), topical (i.e., both skin and mucosal surfaces, including airway surfaces) and transdermal administration. The compositions herein may also be administered via a skin scarification method, or transdermally via a patch or liquid. The compositions may be delivered subdermally in the form of a biodegradable material that releases the compositions over a period of time. The most suitable route in any given case will depend, as is well known in the art, on such factors as the species, age, gender and overall condition of the subject, the nature and severity of the condition being treated and/or

on the nature of the particular composition (i.e., dosage, formulation) that is being administered.

The ARPs can also be present in a formulation of this invention in an immunogenic amount. An "immunogenic amount" is an amount of the infectious alphavirus replicon particles which is sufficient to evoke an immune response in the subject to which the pharmaceutical formulation is administered. An amount of from about 10^4 to about 10^{10}, preferably 10^5 to 10^9, and in particular 10^6 to 10^8 infectious units (IU., as measured by indirect immunofluorescence assay), or ARPs, per dose can be administered to a subject, depending upon the age and species of the subject being treated.

Subjects to which effective and/or immunogenic amounts of the compositions of the present invention are administered include human and animal (e.g., mouse, monkey, guinea pig) subjects.

The vaccine compositions of this invention further comprise combinations of CMV polypeptide expressing ARPs with other CMV polypeptide expressing systems to provide the broadest (i.e., all aspects of the immune response, including those features described hereinabove) cellular and humoral responses possible. In certain embodiments, this can include the use of heterologous prime-boost strategies, in which the ARP compositions are used in combination with one or more of the following: recombinantly produced, purified CMV polypeptides (or immunogenic fragments thereof), naked nucleic acids encoding one or more CMV polypeptides, immunogenic fragments or epitopes, such nucleic acids formulated with lipid-containing moieties, non-alphavirus vectors (e.g., pox vectors, adenoviral vectors, herpes vectors, vesicular stomatitis virus vectors, paramyxoviral vectors, parvovirus vectors, papovavirus vectors, adeno-associated virus vectors and retroviral vectors) expressing one or more CMV immunogens, and other alphavirus vectors expressing one or more CMV immunogens. The viral vectors can be virus-like particles or nucleic acids. The alphavirus vectors can be replicon-containing particles, DNA-based replicon-containing vectors (sometimes referred to as an "ELVIS" system, see, for example, U.S. Patent No. 5,814,482) and/or naked RNA vectors.

Thus, the present invention further provides a method of inducing an immune response to CMV in a subject, comprising administering to the subject an effective amount of

the populations, particles and/or compositions of this invention. Also provided herein is a method of preventing or treating a CMV infection in a subject, comprising administering to the subject an effective amount of a population, particle and/or composition of this invention.

As used herein, an "effective amount" refers to an amount of a compound or composition that is sufficient to produce a desired effect, which can be a therapeutic, prophylactic and/or beneficial effect.

Also as used herein, the terms "treat," "treating" and "treatment" include any type of mechanism, action or activity that results in a change in the medical status of a subject, including an improvement in the condition of the subject (*e.g.*, change or improvement in one or more symptoms and/or clinical parameters), delay in the progression of the condition, prevention or delay of the onset of a disease or illness, etc.

In some embodiments, the present invention provides a method for inducing an immune response to CMV in a subject, comprising: a) priming the subject's immune system by administering to the subject an effective amount of a first immunizing component, which can be, but is not limited to, alphavirus replicon particles encoding CMV immunogens, CMV immunogens, nucleic acid molecules encoding CMV immunogens, a non-alphavirus viral vector encoding CMV immunogens, and any combination thereof; and b) boosting the subject's priming response by administering to the subject an effective amount of a second immunizing component, which can be, but is not limited to, alphavirus replicon particles encoding CMV immunogens, CMV immunogens, nucleic acid molecules encoding CMV immunogens, a non-alphavirus viral vector encoding CMV immunogens, and any combination thereof, wherein the first immunizing component can be different from the second immunizing component and wherein at least the first immunizing component or the second immunizing component is an alphavirus replicon particle encoding CMV immunogens.

In the methods of this invention, the first immunizing component can be a first alphavirus replicon particle and the second immunizing component can be a second alphavirus replicon particle, with the proviso that the first and second alphavirus particles are derived from different alphaviruses.

In other embodiments of the methods of this invention, the first immunizing component can comprise-alphavirus replicon particles encoding cytomegalovirus pp65, IE1 and gB proteins or immunogenic fragments thereof, and the second immunizing component can comprise one or more CMV proteins and/or immunogenic fragments thereof.

In the methods of this invention, the immunizing components can be administered once or more than once (i.e., multiple times). For example, a first immunizing component of this invention and/or a second immunizing component of this invention can be administered one, two, three, four, five, six, seven, eight, nine or ten times at any time interval (e.g., hours, days, weeks, months, years, etc.) and in any of the amounts described herein, which can be the same amount each time or different amounts at different times of administration in any combination. In other embodiments, the administration of the first and second immunizing components can be combined or arranged in any order (e.g., the first and second immunizing components can be administered in an alternating sequence or in any other order).

In some embodiments of the present invention, the first and/or second immunizing component can be administered with an adjuvant. As used herein, "adjuvant" describes a substance, which can be any immunomodulating substance capable of being combined with the polypeptide or nucleic acid vaccine to enhance, improve or otherwise modulate an immune response in a subject without deleterious effect on the subject.

An adjuvant of this invention can be, but is not limited to, for example, an immunostimulatory cytokine (including, but not limited to, GM/CSF, interleukin-2, interleukin-12, interferon-gamma, interleukin-4, tumor necrosis factor-alpha, interleukin-1, hematopoietic factor flt3L, CD40L, B7.1 co-stimulatory molecules and B7.2 co-stimulatory molecules), SYNTEX adjuvant formulation 1 (SAF-1) composed of 5 percent (wt/vol) squalene (DASF, Parsippany, N.J.), 2.5 percent Pluronic, L121 polymer (Aldrich Chemical, Milwaukee), and 0.2 percent polysorbate (Tween 80, Sigma) in phosphate-buffered saline. Suitable adjuvants also include oil-in-water, saponin, an aluminum salt such as aluminum hydroxide gel (alum), aluminum phosphate, or algannmulin, but may also be a salt of calcium, iron or zinc, or may be an insoluble suspension of acylated tyrosine, or acylated sugars, cationically or anionically derivatized polysaccharides, or polyphosphazenes.

Other adjuvants are well known in the art and include QS-21, Freund's adjuvant (complete and incomplete), aluminum hydroxide, N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP), N-acetyl-normuramyl-L-alanyl-D-isoglutamine (CGP 11637, referred to as nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn -glycero-3-hydroxyphosphoryloxy)-ethylamine (CGP 19835A, referred to as MTP-PE) and RIBI, which contains three components extracted from bacteria, monophosphoryl lipid A, trealose dimycolate and cell wall skeleton (MPL+TDM+CWS) in 2% squalene/Tween 80 emulsion.

Additional adjuvants can include, for example, a combination of monophosphoryl lipid A, preferably 3-de-O-acylated monophosphoryl. lipid A (3D-MPL) together with an aluminum salt. An enhanced adjuvant system involves the combination of a monophosphoryl lipid A and a saponin derivative, particularly the combination of QS21 and 3D-MPL as disclosed in PCT publication number WO 94/00153 (the entire contents of which are incorporated herein by reference), or a less reactogenic composition where the QS21 is quenched with cholesterol as disclosed in PCT publication number WO 96/33739 (the entire contents of which are incorporated herein by reference). A particularly potent adjuvant formulation involving QS21 3D-MPL & tocopherol in an oil in water emulsion is described in PCT publication number WO 95/17210 (the entire contents of which are incorporated herein by reference). In addition, the nucleic acid of this invention can include a nucleotide sequence that provides an immunostimulatory signal and/or an adjuvant function, such as CpG sequences. Such CpG sequences, or motifs, are well known in the art.

An adjuvant of this invention, such as, for example, an immunostimulatory cytokine, can be administered before, concurrent with, and/or within a few hours, several hours, and/or 1, 2, 3, 4, 5, 6, 7, 8, 9, and/or 10 days before or after the administration of a composition of this invention to a subject.

Furthermore, any combination of adjuvants, such as immunostimulatory cytokines, can be co-administered to the subject before, after or concurrent with the administration of a composition of this invention. For example, combinations of immunostimulatory cytokines, can consist of two or more immunostimulatory cytokines of this invention, such as GM/CSF, interleukin-2, interleukin-12, interferon-gamma, interleukin-4, tumor necrosis factor-alpha,

interleukin-1, hematopoietic factor flt3L, CD40L, B7.1 co-stimulatory molecules and B7.2 co-stimulatory molecules. The effectiveness of an adjuvant or combination of adjuvants can be determined by measuring the immune response directed produced in response to administration of a composition of this invention to a subject with and without the adjuvant or combination of adjuvants, using standard procedures, as described herein and as known in the art.

In various embodiments of this invention comprising a non-alphavirus viral vector, the non-alphavirus viral vector can be, but is not limited to, a retroviral vector, an adenoviral vector, a poxvirus vector, a Vesicular Stomatitis Virus (VSV) vector or a picornavirus vector, as well as any other non-alphavirus viral vector now known or later identified.

The alphavirus particles employed in the methods of this invention can be particles derived from any alphavirus, such as, for example, Venezuelan Equine Encephalitis virus, S.A.AR86 virus, Semliki Forest virus, Sindbis virus, Ross River virus and any combination thereof. The alphavirus particles of this invention can also comprise elements (e.g., structural proteins/ replicon RNA) from two or more different alphaviruses to produce chimeric alphavirus particles (e.g., a particle comprising a Sindbis virus replicon RNA and VEE structural proteins). The production and testing of such chimeric particles is known in the art.

EXAMPLES

Example 1. Cloning of CMV Genes into VEE Replicon (Figure 1)

Standard molecular biology techniques were used in the cloning of all constructs and their analysis. The VEE replicon vector (Rayner et al.) was modified to introduce additional restriction sites for run-off transcription and ease of cloning. CMV genes coding for pp65 (UL83, strain AD169 (American Type Culture Collection No. VR-538), Immediate Early gene 1 (IE1, UL123, strain AD169), full-length glycoprotein B (gB, UL55, strain Towne (American Type Culture Collection No. VR-977) or C-terminus truncated gB (amino acids 1 – 692, excludes predicted transmembrane domain) were cloned under the control of a subgenomic ("SG") 26S promoter to generate replicons with single or multiple CMV genes.

In addition, a pp65-IE1 fusion construct was made, which was cloned into this modified VEE vector in a similar fashion. Table 1 identifies the various constructs that were made and tested. In some embodiments, a Csp45I restriction site was introduced into the replicon to linearize the vector, as a NotI site was identified in the pp65 coding sequence and NotI was used to linearize this vector for other uses, based on a NotI restriction site in the replicon sequence. In other embodiments, the pp65 coding sequence is modified to remove the NotI restriction site.

Example 2. Production of VEE Replicon Particles expressing CMV Genes

Packaged VEE Replicon Particles (VRP) were obtained after electroporation of CHO or Vero cells with *in vitro*-transcribed replicon and helper RNAs. Cells were maintained in EMEM (Vero) or F12-K (CHO) supplemented with 10% FBS in an atmosphere of 5% CO_2 at 37°C. For electroporation, cells were trypsinized and washed with phosphate buffered saline (PBS). Electroporation was performed using GenePulser Electroporator (Bio-Rad; Hercules, CA) and 0.4 cm cuvettes. After electroporation, the cells were resuspended in growth medium, seeded into tissue culture flasks containing growth medium, and incubated over night. Growth medium containing released VRP was collected, filtered, and tested to confirm the absence of replication competent virus. VRP were then purified by affinity chromatography on HiTrap® heparin HP columns (Amersham, Piscataway, NJ), which are highly cross-linked agarose (6%), activated with N-hydroxysuccinimide and containing porcine heparin as the ligand. The VRP were formulated with 1% Human Serum Albumin and 5% sucrose in phosphate buffered saline.

Example 3. Protein Expression from CMV-expressing VRPs

Expression of nucleic acids to produce CMV proteins was analyzed by SDS-PAGE followed by silver stain (Invitrogen Inc., Carlsbad, CA) or western blot analysis with gene-specific monoclonal (Rumbaugh-Goodwin Institute) or goat polyclonal antibodies. Goat polyclonal antibodies were generated by immunization with purified CMV proteins.

Cells were infected with specified VRP at a multiplicity of infection (moi) of 10 IU/cell and incubated for 18-22 hr. Cell lysates in 0.5% SDS, 0.5% NP-40, 50 mM Tris-HCl,

pH 7.5, 0.1M NaCl, 1mM EDTA were normalized by protein content and 1 μg per lane of total protein was resolved on 4-12% gradient SDS-PAGE (Invitrogen Inc., Carlsbad, CA). Proteins were visualized by silver staining as recommended by manufacturer (Invitrogen Inc., Carlsbad, CA). Silver staining revealed prominent bands of the molecular weight expected for the CMV gene products.

Cells lysates prepared as described were analyzed by western blot with monoclonal or goat polyclonal monospecific antibodies specific for gB protein in reducing or non-reducing conditions. Western blot analysis revealed prominent bands of the molecular weight expected for the CMV gene products.

Example 4. Immunogenicity of VEE Replicon Particles expressing CMV Genes

Groups of 6-week-old female BALB/c mice (Charles River Laboratories, Raleigh, NC) were injected subcutaneously in both rear footpads with a total of 10^6 IU of VRP at weeks 0, 3, and 8. Serum samples were collected by retro-orbital bleed at day -1 (pre-bleed) and weeks 4 and 9. Spleens were harvested at week 15.

Some groups of 12 female BALB/c mice were primed and boosted with gB-VRP or truncated gB-VRP on Days 1 and 22. For the third inoculation given on Day 51, animals in these groups were split in half. Six of the animals in each group (Groups 3A and 4A) received a third VRP inoculation (the same VRP they received for the prime and boost) and the remaining six animals (Groups 3B and 4B) received truncated gB protein adjuvanted with RIBI adjuvant (Corixa Corporation, Seattle, WA) and bacterial endotoxin prepared in saline as their third inoculation. The VRPs were given at an inoculation dose of 1 X 10^6 IU in the subcutaneous rear footpads. For the gB protein inoculations, 50 μg of protein in adjuvant was administered by intraperitoneal injection.

A. Induction of humoral immunity

A CMV neutralization assay was used to evaluate the humoral immune response to CMV gB. CMV neutralization titer was determined by incubating serial dilutions of heat-inactivated sera with a known concentration of CMV (Towne strain) in the presence of 5%

guinea pig complement (CedarLane Laboratories, Hornby, Ontario, Canada). Reduction of CMV infection was determined using a viral neutralization assay, as is standardly known in the art. Neutralization titer (NT_{50}) was defined as 50% reduction in OD_{570} compared to CMV-only control.

Immunization of mice with VRP expressing nucleic acid encoding glycoprotein B (full-length or truncated) resulted in induction of virus-neutralizing antibodies (Figure 2). Neutralizing antibody titers were significantly increased after the second boost. Higher neutralizing antibody titers were seen in Groups 3B and 4B with the addition of protein and adjuvant compared to the groups (Groups 3A and 4A) that got a third dose of VRP instead of protein and adjuvant (See Figure 2, Day 63).

B. Induction of cellular immunity

An IFN-γ ELISPOT assay was used to evaluate the cellular immune response to pp65 and IE1. Splenic lymphocytes were prepared using Lympholyte M density gradient centrifugation after lysis of red blood cells. 96-well ELISPOT IP plates (Millipore, Bedford, MA) were coated with 1 μg anti-mouse IFN-γ mAb/well (MabTech, Mariemont, OH) and blocked with 10% FCS in RPMI-1640 (including supplements). 10^6 lymphocytes/well were plated alone or after mixing with Con A (4 μg/ml) or peptide (10 μg/ml). In all cases, each peptide was tested against known positive and negative lymphocytes and each lymphocyte preparation was tested against known positive and negative peptides. For detection, 0.1 μg of biotinylated anti-mouse IFN-γ (MabTech) was added to each well, followed by incubation with Avidin-Peroxidase Complex (Vector Laboratories, Burlingame, CA), and color development with AEC substrate. Spots were quantified by Zellnet, Inc. (New York, NY) using a Zeiss ELISPOT reader.

Cellular immune response of mice to CMV-expressing VRPs was measured by ELISPOT assay as described above. All constructs induced a robust immune response to pp65 and IE1 proteins (Figure 3).

Example 5. Vaccine "Challenge" in Solid Organ Transplant Recipients

A vaccine of the invention can be tested in "challenge" studies in humans undergoing solid organ transplant surgeries. CMV-seronegative patients on the organ/marrow waiting list are immunized, and then they undergo transplant surgery two weeks to several years after immunization. The "challenge" comes from the transplant itself, since most transplanted organs in the United States (> 60%) come from seropositive donors, and the CMV virus is transmitted via the organ. In addition, these CMV seronegative recipients of CMV seropositive organs are given booster doses of vaccine after the transplant surgery to maintain a sufficient level of immunity to prevent CMV disease. Booster doses are initially given at intervals of every one month to every six months. Patients are monitored and evaluated for at least one year, at monthly or quarterly intervals, and/or after the treatment for signs or symptoms of CMV disease.

Example 6. Vaccination of Bone Marrow Transplant Recipients

A vaccine of this invention can be administered to bone marrow transplant recipients to reduce or eliminate the transmission of HCMV via the donor bone marrow. The risk of disease from such HCMV transmission is particularly high in those seronegative recipients who receive a seropositive bone marrow. In one vaccination protocol, the bone marrow donor is vaccinated with a vaccine of the invention on one or more than one occasion, e.g., at six and two weeks before making the donation; the BMT recipient is vaccinated at intervals (e.g., every one to six months) starting about four weeks to about six months after receiving the transplanted bone marrow. The appearance of CMV viremia is monitored in the transplant recipient.

Example 7. Vaccine "Challenge" in Pregnant Women

A vaccine of the invention can be further tested in women of child-bearing potential who test seronegative for HCMV. Immunization protocols will typically include a priming immunization followed by one or two "booster" immunizations. These women are monitored for pregnancy outcomes, including the rates of CMV infection, symptomatic disease, and delayed sequelae in newborns.

Although the present process has been described with reference to specific details of certain embodiments thereof, it is not intended that such details should be regarded as limitations upon the scope of the invention except as and to the extent that they are included in the accompanying claims.

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Throughout this application, various patents, patent publications and non-patent publications are referenced. The disclosures of these patents and publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

Table 1. Organization of CMV genes in CMV-VRP vaccine candidates.

#	*Designation*	*SG promoter #1*	*SG promoter #2*	*SG promoter #3*
1	VRP-pp65	pp65		
2	VRP-IE1	IE1		
3	VRP-gB	gB		
4	VRP-Tr-gB	Tr-gB		
5	VRP-pp65/IE1	pp65	IE1	
9	VRP-pp65/gB	pp65	gB	
6	VRP-IE1/pp65	IE1	pp65	
11	VRP-pp65/IE1/gB	pp65	IE1	gB
10	VRP-pp65/IE1/Tr-gB	pp65	1E1	Tr-gB
7	VRP-pp65-IE1 fusion	pp65-IE1 fusion		
8	VRP-pp65-IE1 fusion/Tr-gB	pp65-IE1 fusion	Tr-gB	

What is claimed is:

1. A population of alphavirus replicon particles wherein said particles comprise alphavirus replicon RNAs, wherein a first replicon RNA comprises nucleic acid encoding cytomegalovirus pp65 and IE1 proteins or immunogenic fragments thereof, and a second replicon RNA comprises nucleic acid encoding cytomegalovirus gB protein or an immunogenic fragment thereof, and wherein each of the first and second replicon RNAs is contained within a separate alphavirus replicon particle.

2. A population of alphavirus replicon particles wherein said particles comprise a replicon RNA which comprises a regulatory cassette that directs transcription and translation of a nucleic acid encoding cytomegalovirus pp65 and IE1 proteins, or immunogenic fragments thereof.

3. The population of claim 2, wherein the nucleic acid encodes a fusion protein of pp65 and IE1.

4. The population of claim 1, wherein the first replicon RNA directs transcription and translation of the nucleic acid encoding cytomegalovirus pp65 and IE1 proteins or immunogenic fragments thereof from two separate regulatory cassettes, whereby a first regulatory cassette directs transcription and translation of the CMV pp65-encoding nucleic acid and a second regulatory cassette directs replication and translation of the CMV IE1-encoding nucleic acid.

5. The population of claim 1, wherein the first replicon RNA encodes a fusion protein of pp65 and IE1.

6. The population of claim 4, wherein the first and second regulatory cassettes are the same.

7. The population of claim 2, wherein the regulatory cassette is an alphavirus subgenomic promoter.

8. The population of claim 1 wherein the nucleic acid encoding cytomegalovirus gB protein or an immunogenic fragment thereof encodes a CMV gB protein or immunogenic fragment thereof wherein the transmembrane domain has been deleted.

9. The population of claim 4, wherein the first and second regulatory cassettes are different.

10. The population of claim 9, wherein the first and/or second regulatory cassette comprises (i) an alphavirus subgenomic promoter to direct transcription, and (ii) an IRES element to direct translation.

11. The population of claim 10 wherein the replicon RNA of each particle further encodes a CMV gB protein, or immunogenic fragment thereof and wherein production of the CMV gB protein is under the control of a separate, third regulatory cassette.

12. A population of alphavirus replicon particles wherein the particles comprise an alphavirus replicon RNA comprising nucleic acid encoding a CMV polypeptide selected from the group consisting of pp65, IE1, and gB, immunogenic fragments thereof or any combination thereof.

13. A population of alphavirus replicon particles, wherein the particles comprise an alphavirus replicon RNA comprising nucleic acid encoding-cytomegalovirus pp65 and gB proteins, or immunogenic fragments thereof.

14. The population of claim 13, wherein the expression of the nucleic acid encoding cytomegalovirus pp65 and the expression of the nucleic acid encoding gB protein is controlled by separate regulatory cassettes.

15. A population of alphavirus replicon particles comprising an alphavirus replicon RNA, wherein the replicon RNA of each particle comprises a first nucleic acid encoding cytomegalovirus pp65 protein or an immunogenic fragment thereof and a second nucleic acid encoding cytomegalovirus IE1 protein or an immunogenic fragment thereof, and wherein the expression of the first and second nucleic acid is controlled by separate regulatory cassettes.

16. A composition comprising the population of claim 1 in a pharmaceutically acceptable carrier.

17. A method of inducing an immune response to CMV in a subject, comprising administering to the subject an effective amount of the population of claim 1.

18. The method of claim 17, wherein the population is administered multiple times.

19. A method for inducing an immune response to CMV in a subject, comprising
a) priming the subject's immune system by administering to the subject an effective amount of a first immunizing component selected from the group consisting of:
alphavirus replicon particles encoding CMV immunogens,
CMV immunogens,
nucleic acid molecules encoding CMV immunogens,
a non-alphavirus viral vector encoding CMV immunogens, and
any combination thereof; and
b) boosting the subject's priming response by administering to the subject an effective amount of a second immunizing component selected from the group consisting of:
alphavirus replicon particles encoding CMV immunogens,
CMV immunogens,
nucleic acid molecules encoding CMV immunogens,
a non-alphavirus viral vector encoding CMV immunogens, and
any combination thereof,
wherein the first immunizing component is different from the second immunizing component and wherein at least the first immunizing component or the second immunizing component is an alphavirus replicon particle encoding CMV immunogens.

20. The method of claim 19 wherein the first immunizing component is a first alphavirus replicon particle and the second immunizing component is a second alphavirus replicon particle, with the proviso that the first and second alphavirus particles are derived from different alphaviruses.

21. The method of claim 19 further comprising administering the first and/or second immunizing component multiple times.

22. The method of claim 19 wherein the first immunizing component comprises alphavirus replicon particles encoding cytomegalovirus pp65, IE1 and gB proteins or immunogenic fragments thereof, and wherein the second immunizing component comprises one or more CMV proteins and/or immunogenic fragments thereof.

23. The method of claim 22 wherein the CMV protein is a truncated gB protein.

24. The method of claim 19 wherein the first and/or second immunizing component is administered with an adjuvant.

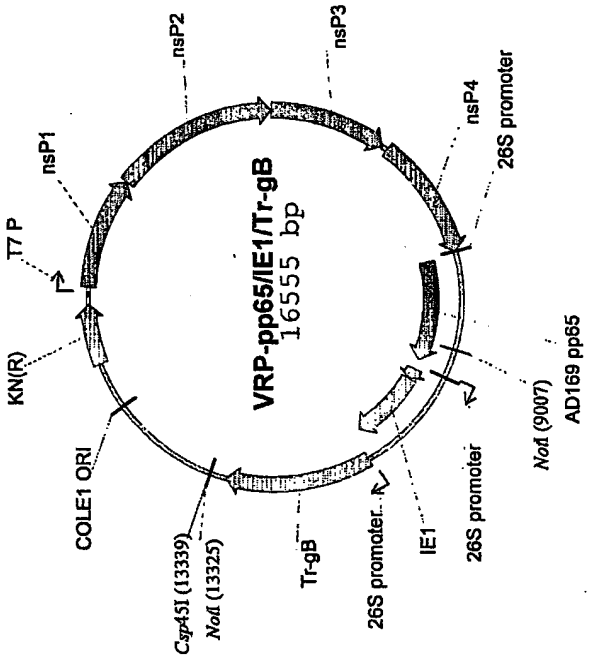
25. The method of claim 24 wherein the adjuvant is selected from the group consisting of aluminum salts, oil-in-water, saponin, cytokines, oligonucleotides encoding immunostimulatory signals and any combination thereof.

26. The method of claim 19 wherein the non-alphavirus viral vector is selected from the group consisting of a retroviral vector, an adenoviral vector, a poxvirus vector, a Vesicular Stomatitis Virus (VSV) vector and a picornavirus vector.

27. The method of claim 19, wherein the alphavirus replicon particles are selected from the group consisting of particles derived from Venezuelan Equine Encephalitis virus, S.A.AR86 virus, Semliki Forest virus, Sindbis virus, Ross River virus and any combination thereof.

28. The method of claim 19, wherein the alphavirus replicon particles comprise elements from two or more alphaviruses.

Fig. 1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KN(R)
COLE1 ORI
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NotI (13325)
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IE1
26S promoter
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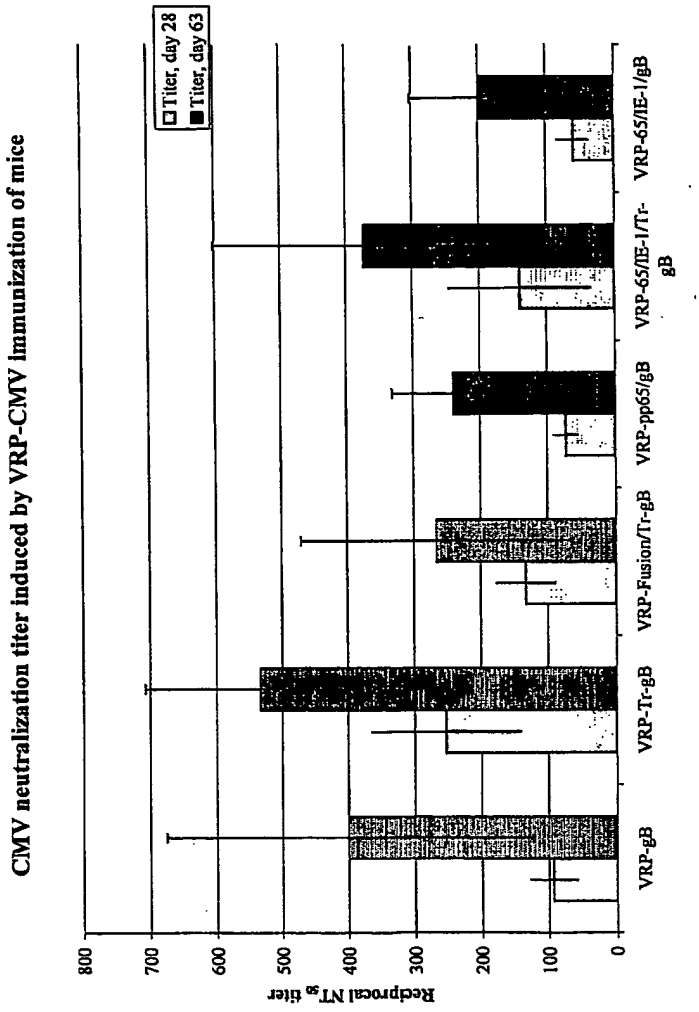
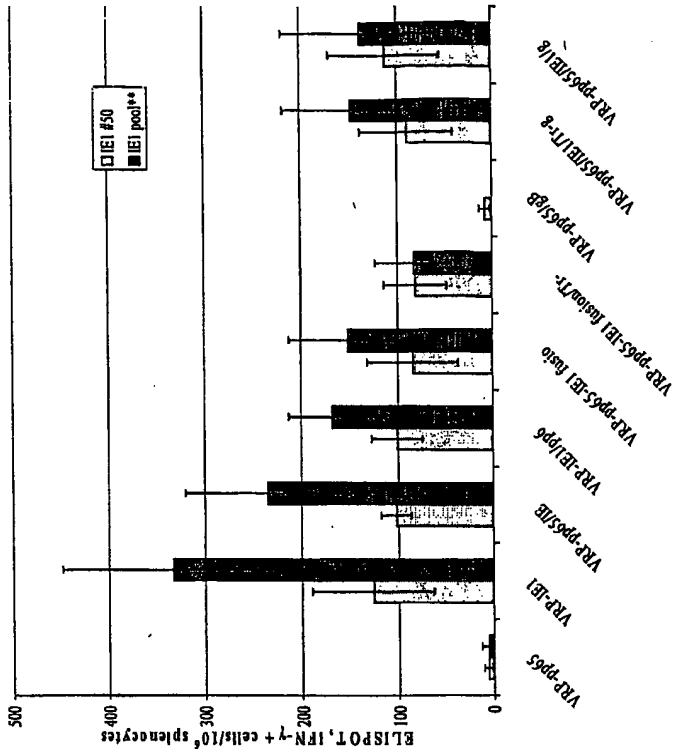
AD169 pp65

Fig. 2



Figs. 3A-B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IEi pool**
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300
400
500
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VRP-ppb5/gB
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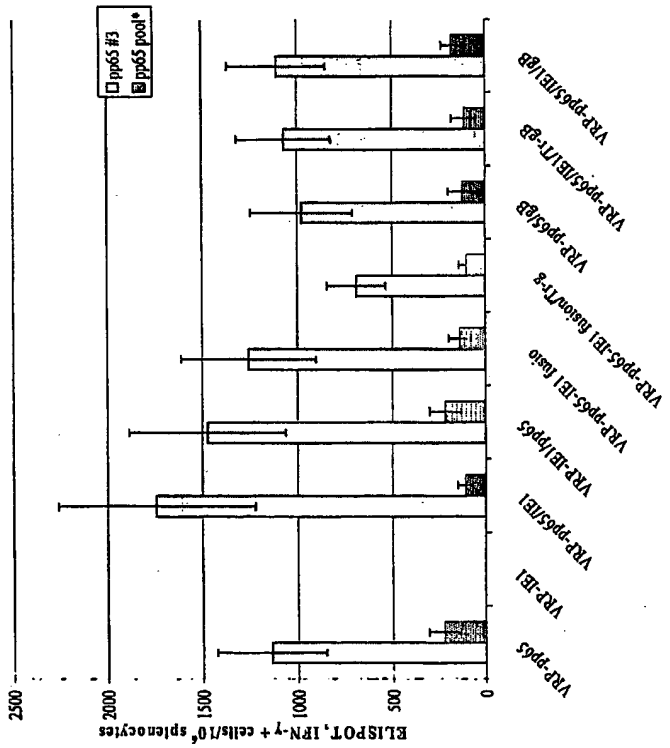
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1500
2000
2500
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VRP-ppb5/IE1/T-gB
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VRP-ppb5-IE1 fusion/T-8
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VRP-IE1
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<110> Alphavax, Inc.
Chulay, Jeffrey D.
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ata gca ggc acg gtt ttc gtg gct tat cat agg gac agc tat gaa aac      624
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Phe Met His Val Thr Leu Gly Ser Asp Val Glu Glu Asp Leu Thr Met
                245                 250                 255

acc cgc aac ccg caa ccc ttc atg cgc ccc cac gag cgc aac ggc ttt      816
Thr Arg Asn Pro Gln Pro Phe Met Arg Pro His Glu Arg Asn Gly Phe
            260                 265                 270

acg gtg ttg tgt ccc aaa aat atg ata atc aaa ccg ggc aag atc tcg      864
Thr Val Leu Cys Pro Lys Asn Met Ile Ile Lys Pro Gly Lys Ile Ser
        275                 280                 285

cac atc atg ctg gat gtg gct ttt acc tca cac gag cat ttt ggg ctg      912
His Ile Met Leu Asp Val Ala Phe Thr Ser His Glu His Phe Gly Leu
    290                 295                 300

ctg tgt ccc aag agc atc ccg ggc ctg agc atc tca ggt aac ctg ttg      960
Leu Cys Pro Lys Ser Ile Pro Gly Leu Ser Ile Ser Gly Asn Leu Leu
305                 310                 315                 320

atg aac ggg cag cag atc ttc ctg gag gta caa gcc ata cgc gag acc     1008
Met Asn Gly Gln Gln Ile Phe Leu Glu Val Gln Ala Ile Arg Glu Thr
                325                 330                 335

gtg gaa ctg cgt cag tac gat ccc gtg gct gcg ctc ttc ttt ttc gat     1056
Val Glu Leu Arg Gln Tyr Asp Pro Val Ala Ala Leu Phe Phe Phe Asp
            340                 345                 350

atc gac ttg ctg ctg cag cgc ggg cct cag tac agc gag cac ccc acc     1104
Ile Asp Leu Leu Leu Gln Arg Gly Pro Gln Tyr Ser Glu His Pro Thr
        355                 360                 365

ttc acc agc cag tat cgc atc cag ggc aag ctt gag tac cga cac acc     1152
Phe Thr Ser Gln Tyr Arg Ile Gln Gly Lys Leu Glu Tyr Arg His Thr
    370                 375                 380

tgg gac cgg cac gac gag ggt gcc gcc cag ggc gac gac gac gtc tgg     1200
Trp Asp Arg His Asp Glu Gly Ala Ala Gln Gly Asp Asp Asp Val Trp
385                 390                 395                 400

acc agc gga tcg gac tcc gac gaa gaa ctc gta acc acc gag cgc aag     1248
Thr Ser Gly Ser Asp Ser Asp Glu Glu Leu Val Thr Thr Glu Arg Lys
                405                 410                 415

acg ccc cgc gtc acc ggc ggc ggc gcc atg gcg ggc gcc tcc act tcc     1296
Thr Pro Arg Val Thr Gly Gly Gly Ala Met Ala Gly Ala Ser Thr Ser
            420                 425                 430
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gcg ggc cgc aaa cgc aaa tca gca tcc tcg gcg acg gcg tgc acg tcg      1344
Ala Gly Arg Lys Arg Lys Ser Ala Ser Ser Ala Thr Ala Cys Thr Ser
        435                 440                 445

ggc gtt atg aca cgc ggc cgc ctt aag gcc gag tcc acc gtc gcg ccc      1392
Gly Val Met Thr Arg Gly Arg Leu Lys Ala Glu Ser Thr Val Ala Pro
    450                 455                 460

gaa gag gac acc gac gag gat tcc gac aac gaa atc cac aat ccg gcc      1440
Glu Glu Asp Thr Asp Glu Asp Ser Asp Asn Glu Ile His Asn Pro Ala
465                 470                 475                 480

gtg ttc acc tgg ccg ccc tgg cag gcc ggc atc ctg gcc cgc aac ctg      1488
Val Phe Thr Trp Pro Pro Trp Gln Ala Gly Ile Leu Ala Arg Asn Leu
                485                 490                 495

gtg ccc atg gtg gct acg gtt cag ggt cag aat ctg aag tac cag gaa      1536
Val Pro Met Val Ala Thr Val Gln Gly Gln Asn Leu Lys Tyr Gln Glu
            500                 505                 510

ttc ttc tgg gac gcc aac gac atc tac cgc atc ttc gcc gaa ttg gaa      1584
Phe Phe Trp Asp Ala Asn Asp Ile Tyr Arg Ile Phe Ala Glu Leu Glu
        515                 520                 525

ggc gta tgg cag ccc gct gcg caa ccc aaa cgt cgc cgc cac cgg caa      1632
Gly Val Trp Gln Pro Ala Ala Gln Pro Lys Arg Arg Arg His Arg Gln
    530                 535                 540

gac gcc ttg ccc ggg cca tgc atc gcc tcg acg ccc aaa aag cac cga      1680
Asp Ala Leu Pro Gly Pro Cys Ile Ala Ser Thr Pro Lys Lys His Arg
545                 550                 555                 560

ggt gag tcc tct gcc aag aga aag atg gac cct gat aat cct gac gag      1728
Gly Glu Ser Ser Ala Lys Arg Lys Met Asp Pro Asp Asn Pro Asp Glu
                565                 570                 575

ggc cct tcc tcc aag gtg cca cgg ccc gag aca ccc gtg acc aag gcc      1776
Gly Pro Ser Ser Lys Val Pro Arg Pro Glu Thr Pro Val Thr Lys Ala
            580                 585                 590

acg acg ttc ctg cag act atg ttg agg aag gag gtt aac agt cag ctg      1824
Thr Thr Phe Leu Gln Thr Met Leu Arg Lys Glu Val Asn Ser Gln Leu
        595                 600                 605

agt ctg gga gac ccg ctg ttt cca gag ttg gcc gaa gaa tcc ctc aaa      1872
Ser Leu Gly Asp Pro Leu Phe Pro Glu Leu Ala Glu Glu Ser Leu Lys
    610                 615                 620

act ttt gaa caa gtg acc gag gat tgc aac gag aac ccc gag aaa gat      1920
Thr Phe Glu Gln Val Thr Glu Asp Cys Asn Glu Asn Pro Glu Lys Asp
625                 630                 635                 640

gtc ctg gca gaa ctc gtc aaa cag att aag gtt cga gtg gac atg gtg      1968
Val Leu Ala Glu Leu Val Lys Gln Ile Lys Val Arg Val Asp Met Val
                645                 650                 655
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cgg cat aga atc aag gag cac atg ctg aaa aaa tat acc cag acg gaa     2016
Arg His Arg Ile Lys Glu His Met Leu Lys Lys Tyr Thr Gln Thr Glu
            660                 665                 670

gag aaa ttc act ggc gcc ttt aat atg atg gga gga tgt ttg cag aat     2064
Glu Lys Phe Thr Gly Ala Phe Asn Met Met Gly Gly Cys Leu Gln Asn
        675                 680                 685

gcc tta gat atc tta gat aag gtt cat gag cct ttc gag gag atg aag     2112
Ala Leu Asp Ile Leu Asp Lys Val His Glu Pro Phe Glu Glu Met Lys
    690                 695                 700

tgt att ggg cta act atg cag agc atg tat gag aac tac att gta cct     2160
Cys Ile Gly Leu Thr Met Gln Ser Met Tyr Glu Asn Tyr Ile Val Pro
705                 710                 715                 720

gag gat aag cgg gag atg tgg atg gct tgt att aag gag ctg cat gat     2208
Glu Asp Lys Arg Glu Met Trp Met Ala Cys Ile Lys Glu Leu His Asp
                725                 730                 735

gtg agc aag ggc gcc gct aac aag ttg ggg ggt gca ctg cag gct aag     2256
Val Ser Lys Gly Ala Ala Asn Lys Leu Gly Gly Ala Leu Gln Ala Lys
            740                 745                 750

gcc cgt gct aaa aag gat gaa ctt agg aga aag atg atg tat atg tgc     2304
Ala Arg Ala Lys Lys Asp Glu Leu Arg Arg Lys Met Met Tyr Met Cys
        755                 760                 765

tac agg aat ata gag ttc ttt acc aag aac tca gcc ttc cct aag acc     2352
Tyr Arg Asn Ile Glu Phe Phe Thr Lys Asn Ser Ala Phe Pro Lys Thr
    770                 775                 780

acc aat ggc tgc agt cag gcc atg gcg gca ctg cag aac ttg cct cag     2400
Thr Asn Gly Cys Ser Gln Ala Met Ala Ala Leu Gln Asn Leu Pro Gln
785                 790                 795                 800

tgc tcc cct gat gag att atg gct tat gcc cag aaa ata ttt aag att     2448
Cys Ser Pro Asp Glu Ile Met Ala Tyr Ala Gln Lys Ile Phe Lys Ile
                805                 810                 815

ttg gat gag gag aga gac aag gtg ctc acg cac att gat cac ata ttt     2496
Leu Asp Glu Glu Arg Asp Lys Val Leu Thr His Ile Asp His Ile Phe
            820                 825                 830

atg gat atc ctc act aca tgt gtg gaa aca atg tgt aat gag tac aag     2544
Met Asp Ile Leu Thr Thr Cys Val Glu Thr Met Cys Asn Glu Tyr Lys
        835                 840                 845

gtc act agt gac gct tgt atg atg acc atg tac ggg ggc atc tct ctc     2592
Val Thr Ser Asp Ala Cys Met Met Thr Met Tyr Gly Gly Ile Ser Leu
    850                 855                 860

tta agt gag ttc tgt cgg gtg ctg tgc tgc tat gtc tta gag gag act     2640
Leu Ser Glu Phe Cys Arg Val Leu Cys Cys Tyr Val Leu Glu Glu Thr
865                 870                 875                 880

agt gtg atg ctg gcc aag cgg cct ctg ata acc aag cct gag gtt atc     2688
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```
Ser Val Met Leu Ala Lys Arg Pro Leu Ile Thr Lys Pro Glu Val Ile
                885                 890                 895

agt gta atg aag cgc cgc att gag gag atc tgc atg aag gtc ttt gcc     2736
Ser Val Met Lys Arg Arg Ile Glu Glu Ile Cys Met Lys Val Phe Ala
            900                 905                 910

cag tac att ctg ggg gcc gat cct ctg aga gtc tgc tct cct agt gtg     2784
Gln Tyr Ile Leu Gly Ala Asp Pro Leu Arg Val Cys Ser Pro Ser Val
        915                 920                 925

gat gac cta cgg gcc gtc gcc gag gag tca gat gag gaa gag gct att     2832
Asp Asp Leu Arg Ala Val Ala Glu Glu Ser Asp Glu Glu Glu Ala Ile
    930                 935                 940

gta gcc tac act ttg gcc acc gct ggt gtc agc tcc tct gat tct ctg     2880
Val Ala Tyr Thr Leu Ala Thr Ala Gly Val Ser Ser Ser Asp Ser Leu
945                 950                 955                 960

gtg tca ccc cca gag tcc cct gta ccc gcg act atc cct ctg tcc tca     2928
Val Ser Pro Pro Glu Ser Pro Val Pro Ala Thr Ile Pro Leu Ser Ser
                965                 970                 975

gta att gtg gct gag aac agt gat cag gaa gaa agt gag cag agt gat     2976
Val Ile Val Ala Glu Asn Ser Asp Gln Glu Glu Ser Glu Gln Ser Asp
            980                 985                 990

gag gaa gag gag gag ggt gct cag  gag gag cgg gag gac  act gtg tct   3024
Glu Glu Glu Glu Glu Gly Ala Gln  Glu Glu Arg Glu Asp  Thr Val Ser
        995                 1000                1005

gtc aag  tct gag cca gtg tct  gag ata gag gaa gtt  gcc cca gag      3069
Val Lys  Ser Glu Pro Val Ser  Glu Ile Glu Glu Val  Ala Pro Glu
    1010                1015                1020

gaa gag  gag gat ggt gct gag  gaa ccc acc gcc tct  gga ggc aag      3114
Glu Glu  Glu Asp Gly Ala Glu  Glu Pro Thr Ala Ser  Gly Gly Lys
    1025                1030                1035

agc acc  cac cct atg gtg act  aga agc aag gct gac  cag taa          3156
Ser Thr  His Pro Met Val Thr  Arg Ser Lys Ala Asp  Gln
    1040                1045                1050


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<212>  PRT
<213>  Artificial

<220>
<223>  hCMV pp65-IE1 fusion

<400>  4

Met Glu Ser Arg Gly Arg Arg Cys Pro Glu Met Ile Ser Val Leu Gly
1               5                   10                  15
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Pro Ile Ser Gly His Val Leu Lys Ala Val Phe Ser Arg Gly Asp Thr
20 25 30

Pro Val Leu Pro His Glu Thr Arg Leu Leu Gln Thr Gly Ile His Val
35 40 45

Arg Val Ser Gln Pro Ser Leu Ile Leu Val Ser Gln Tyr Thr Pro Asp
50 55 60

Ser Thr Pro Cys His Arg Gly Asp Asn Gln Leu Gln Val Gln His Thr
65 70 75 80

Tyr Phe Thr Gly Ser Glu Val Glu Asn Val Ser Val Asn Val His Asn
85 90 95

Pro Thr Gly Arg Ser Ile Cys Pro Ser Gln Glu Pro Met Ser Ile Tyr
100 105 110

Val Tyr Ala Leu Pro Leu Lys Met Leu Asn Ile Pro Ser Ile Asn Val
115 120 125

His His Tyr Pro Ser Ala Ala Glu Arg Lys His Arg His Leu Pro Val
130 135 140

Ala Asp Ala Val Ile His Ala Ser Gly Lys Gln Met Trp Gln Ala Arg
145 150 155 160

Leu Thr Val Ser Gly Leu Ala Trp Thr Arg Gln Gln Asn Gln Trp Lys
165 170 175

Glu Pro Asp Val Tyr Tyr Thr Ser Ala Phe Val Phe Pro Thr Lys Asp
180 185 190

Val Ala Leu Arg His Val Val Cys Ala His Glu Leu Val Cys Ser Met
195 200 205

Glu Asn Thr Arg Ala Thr Lys Met Gln Val Ile Gly Asp Gln Tyr Val
210 215 220

Lys Val Tyr Leu Glu Ser Phe Cys Glu Asp Val Pro Ser Gly Lys Leu
225 230 235 240

```
Phe Met His Val Thr Leu Gly Ser Asp Val Glu Glu Asp Leu Thr Met
                245                 250                 255

Thr Arg Asn Pro Gln Pro Phe Met Arg Pro His Glu Arg Asn Gly Phe
            260                 265                 270

Thr Val Leu Cys Pro Lys Asn Met Ile Ile Lys Pro Gly Lys Ile Ser
        275                 280                 285

His Ile Met Leu Asp Val Ala Phe Thr Ser His Glu His Phe Gly Leu
    290                 295                 300

Leu Cys Pro Lys Ser Ile Pro Gly Leu Ser Ile Ser Gly Asn Leu Leu
305                 310                 315                 320

Met Asn Gly Gln Gln Ile Phe Leu Glu Val Gln Ala Ile Arg Glu Thr
                325                 330                 335

Val Glu Leu Arg Gln Tyr Asp Pro Val Ala Ala Leu Phe Phe Phe Asp
            340                 345                 350

Ile Asp Leu Leu Leu Gln Arg Gly Pro Gln Tyr Ser Glu His Pro Thr
        355                 360                 365

Phe Thr Ser Gln Tyr Arg Ile Gln Gly Lys Leu Glu Tyr Arg His Thr
    370                 375                 380

Trp Asp Arg His Asp Glu Gly Ala Ala Gln Gly Asp Asp Asp Val Trp
385                 390                 395                 400

Thr Ser Gly Ser Asp Ser Asp Glu Glu Leu Val Thr Thr Glu Arg Lys
                405                 410                 415

Thr Pro Arg Val Thr Gly Gly Gly Ala Met Ala Gly Ala Ser Thr Ser
            420                 425                 430

Ala Gly Arg Lys Arg Lys Ser Ala Ser Ser Ala Thr Ala Cys Thr Ser
        435                 440                 445

Gly Val Met Thr Arg Gly Arg Leu Lys Ala Glu Ser Thr Val Ala Pro
    450                 455                 460

Glu Glu Asp Thr Asp Glu Asp Ser Asp Asn Glu Ile His Asn Pro Ala
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465 470 475 480

Val Phe Thr Trp Pro Pro Trp Gln Ala Gly Ile Leu Ala Arg Asn Leu
485 490 495

Val Pro Met Val Ala Thr Val Gln Gly Gln Asn Leu Lys Tyr Gln Glu
500 505 510

Phe Phe Trp Asp Ala Asn Asp Ile Tyr Arg Ile Phe Ala Glu Leu Glu
515 520 525

Gly Val Trp Gln Pro Ala Ala Gln Pro Lys Arg Arg Arg His Arg Gln
530 535 540

Asp Ala Leu Pro Gly Pro Cys Ile Ala Ser Thr Pro Lys Lys His Arg
545 550 555 560

Gly Glu Ser Ser Ala Lys Arg Lys Met Asp Pro Asp Asn Pro Asp Glu
565 570 575

Gly Pro Ser Ser Lys Val Pro Arg Pro Glu Thr Pro Val Thr Lys Ala
580 585 590

Thr Thr Phe Leu Gln Thr Met Leu Arg Lys Glu Val Asn Ser Gln Leu
595 600 605

Ser Leu Gly Asp Pro Leu Phe Pro Glu Leu Ala Glu Glu Ser Leu
610 615 620

Thr Phe Glu Gln Val Thr Glu Asp Cys Asn Glu Asn Glu Glu Lys Asp
625 630 635 640

Val Leu Ala Glu Leu Val Lys Gln Il Lys Val Arg Val Asp Met Val
645 650 655

Arg His Arg Ile Lys Glu His Met Leu Lys Lys Tyr Thr Gln Thr Glu
660 665 670

Glu Lys Phe Thr Gly Ala Phe Asn Met Met Gly Gly Cys Leu Gln Asn
675 680 685

Ala Leu Asp Ile Leu Asp Lys Val His Glu Pro Phe Glu Glu Met Lys
690 695 700

Cys Ile Gly Leu Thr Met Gln Ser Met Tyr Glu Asn Tyr Ile Val Pro
705 710 715 720

Glu Asp Lys Arg Glu Met Trp Met Ala Cys Ile Lys Glu Leu His Asp
725 730 735

Val Ser Lys Gly Ala Ala Asn Lys Leu Gly Gly Ala Leu Gln Ala Lys
740 745 750

Ala Arg Ala Lys Lys Asp Glu Leu Arg Arg Lys Met Met Tyr Met Cys
755 760 765

Tyr Arg Asn Ile Glu Phe Phe Thr Lys Asn Ser Ala Phe Pro Lys Thr
770 775 780

Thr Asn Gly Cys Ser Gln Ala Met Ala Ala Leu Gln Asn Leu Pro Gln
785 790 795 800

Cys Ser Pro Asp Glu Ile Met Ala Tyr Ala Gln Lys Ile Phe Lys Ile
805 810 815

Leu Asp Glu Glu Arg Asp Lys Val Leu Thr His Ile Asp His Ile Phe
820 825 830

Met Asp Ile Leu Thr Thr Cys Val Glu Thr Met Cys Asn Glu Tyr Lys
835 840 845

Val Thr Ser Asp Ala Cys Met Met Thr Met Tyr Gly Gly Ile Ser Leu
850 855 860

Leu Ser Glu Phe Cys Arg Val Leu Cys Cys Tyr Val Leu Glu Glu Thr
865 870 875 880

Ser Val Met Leu Ala Lys Arg Pro Leu Ile Thr Lys Pro Glu Val Ile
885 890 895

Ser Val Met Lys Arg Arg Ile Glu Glu Ile Cys Met Lys Val Phe Ala
900 905 910

Gln Tyr Ile Leu Gly Ala Asp Pro Leu Arg Val Cys Ser Pro Ser Val
915 920 925

```
Asp Asp Leu Arg Ala Val Ala Glu Glu Ser Asp Glu Glu Glu Ala Ile
    930                 935                 940

Val Ala Tyr Thr Leu Ala Thr Ala Gly Val Ser Ser Ser Asp Ser Leu
945                 950                 955                 960

Val Ser Pro Pro Glu Ser Pro Val Pro Ala Thr Ile Pro Leu Ser Ser
                965                 970                 975

Val Ile Val Ala Glu Asn Ser Asp Gln Glu Glu Ser Glu Gln Ser Asp
            980                 985                 990

Glu Glu Glu Glu Glu Gly Ala Gln  Glu Glu Arg Glu Asp  Thr Val Ser
        995                 1000                1005

Val Lys  Ser Glu Pro Val Ser  Glu Ile Glu Glu Val  Ala Pro Glu
    1010                1015                1020

Glu Glu  Glu Asp Gly Ala Glu  Glu Pro Thr Ala Ser  Gly Gly Lys
    1025                1030                1035

Ser Thr  His Pro Met Val Thr  Arg Ser Lys Ala Asp  Gln
    1040                1045                1050
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<210> 5
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<212> PRT
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Met Glu Ser Arg Gly Arg Arg Cys Pro Glu Met Ile Ser Val Leu
1               5                   10                  15
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<210> 6
<211> 15
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<213> Human cytomegalovirus

<400> 6

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Gly Arg Arg Cys Pro Glu Met Ile Ser Val Leu Gly Pro Ile Ser
1               5                   10                  15
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<210> 7
<211> 15

<212> PRT
<213> Human cytomegalovirus

<400> 7

Pro Glu Met Ile Ser Val Leu Gly Pro Ile Ser Gly His Val Leu
1 5 10 15

<210> 8
<211> 15
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<400> 8

Ser Val Leu Gly Pro Ile Ser Gly His Val Leu Lys Ala Val Phe
1 5 10 15

<210> 9
<211> 15
<212> PRT
<213> Human cytomegalovirus

<400> 9

Pro Ile Ser Gly His Val Leu Lys Ala Val Phe Ser Arg Gly Asp
1 5 10 15

<210> 10
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<212> PRT
<213> Human cytomegalovirus

<400> 10

His Val Leu Lys Ala Val Phe Ser Arg Gly Asp Thr Pro Val Leu
1 5 10 15

<210> 11
<211> 15
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<400> 11

Ala Val Phe Ser Arg Gly Asp Thr Pro Val Leu Pro His Glu Thr
1 5 10 15

<210> 12
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<400> 12

Arg Gly Asp Thr Pro Val Leu Pro His Glu Thr Arg Leu Leu Gln
1 5 10 15

<210> 13
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<400> 13

Pro Val Leu Pro His Glu Thr Arg Leu Leu Gln Thr Gly Ile His
1 5 10 15

<210> 14
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<400> 14

His Glu Thr Arg Leu Leu Gln Thr Gly Ile His Val Arg Val Ser
1 5 10 15

<210> 15
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<400> 15

Leu Leu Gln Thr Gly Ile His Val Arg Val Ser Gln Pro Ser Leu
1 5 10 15

<210> 16
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<400> 16

Gly Ile His Val Arg Val Ser Gln Pro Ser Leu Ile Leu Val Ser
1 5 10 15

<210> 17
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<400> 17

Arg Val Ser Gln Pro Ser Leu Ile Leu Val Ser Gln Tyr Thr Pro
1 5 10 15

<210> 18
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<400> 18

Pro Ser Leu Ile Leu Val Ser Gln Tyr Thr Pro Asp Ser Thr Pro
1 5 10 15

<210> 19
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<400> 19

Leu Val Ser Gln Tyr Thr Pro Asp Ser Thr Pro Cys His Arg Gly
1 5 10 15

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<400> 20

Tyr Thr Pro Asp Ser Thr Pro Cys His Arg Gly Asp Asn Gln Leu
1 5 10 15

<210> 21
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<400> 21

Ser Thr Pro Cys His Arg Gly Asp Asn Gln Leu Gln Val Gln His
1 5 10 15

<210> 22
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<400> 22

His Arg Gly Asp Asn Gln Leu Gln Val Gln His Thr Tyr Phe Thr

1 5 10 15

<210> 23
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Asn Gln Leu Gln Val Gln His Thr Tyr Phe Thr Gly Ser Glu Val
1 5 10 15

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<400> 24

Val Gln His Thr Tyr Phe Thr Gly Ser Glu Val Glu Asn Val Ser
1 5 10 15

<210> 25
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Tyr Phe Thr Gly Ser Glu Val Glu Asn Val Ser Val Asn Val His
1 5 10 15

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<400> 26

Ser Glu Val Glu Asn Val Ser Val Asn Val His Asn Pro Thr Gly
1 5 10 15

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Asn Val Ser Val Asn Val His Asn Pro Thr Gly Arg Ser Ile Cys
1 5 10 15

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<400> 28

Asn Val His Asn Pro Thr Gly Arg Ser Ile Cys Pro Ser Gln Glu
1 5 10 15

<210> 29
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<400> 29

Pro Thr Gly Arg Ser Ile Cys Pro Ser Gln Glu Pro Met Ser Ile
1 5 10 15

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<400> 30

Ser Ile Cys Pro Ser Gln Glu Pro Met Ser Ile Tyr Val Tyr Ala
1 5 10 15

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<400> 31

Ser Gln Glu Pro Met Ser Ile Tyr Val Tyr Ala Leu Pro Leu Lys
1 5 10 15

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<400> 32

Met Ser Ile Tyr Val Tyr Ala Leu Pro Leu Lys Met Leu Asn Ile
1 5 10 15

<210> 33

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<400> 33

Val Tyr Ala Leu Pro Leu Lys Met Leu Asn Ile Pro Ser Ile Asn
1 5 10 15

<210> 34
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<400> 34

Pro Leu Lys Met Leu Asn Ile Pro Ser Ile Asn Val His His Tyr
1 5 10 15

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<400> 35

Leu Asn Ile Pro Ser Ile Asn Val His His Tyr Pro Ser Ala Ala
1 5 10 15

<210> 36
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<400> 36

Ser Ile Asn Val His His Tyr Pro Ser Ala Ala Glu Arg Lys His
1 5 10 15

<210> 37
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<400> 37

His His Tyr Pro Ser Ala Ala Glu Arg Lys His Arg His Leu Pro
1 5 10 15

<210> 38
<211> 15
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<213> Human cytomegalovirus

<400> 38

Ser Ala Ala Glu Arg Lys His Arg His Leu Pro Val Ala Asp Ala
1 5 10 15

<210> 39
<211> 15
<212> PRT
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<400> 39

Arg Lys His Arg His Leu Pro Val Ala Asp Ala Val Ile His Ala
1 5 10 15

<210> 40
<211> 15
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<400> 40

His Leu Pro Val Ala Asp Ala Val Ile His Ala Ser Gly Lys Gln
1 5 10 15

<210> 41
<211> 15
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<400> 41

Ala Asp Ala Val Ile His Ala Ser Gly Lys Gln Met Trp Gln Ala
1 5 10 15

<210> 42
<211> 15
<212> PRT
<213> Human cytomegalovirus

<400> 42

Ile His Ala Ser Gly Lys Gln Met Trp Gln Ala Arg Leu Thr Val
1 5 10 15

<210> 43
<211> 15
<212> PRT
<213> Human cytomegalovirus

<400> 43

Gly Lys Gln Met Trp Gln Ala Arg Leu Thr Val Ser Gly Leu Ala
1 5 10 15

<210> 44
<211> 15
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<400> 44

Trp Gln Ala Arg Leu Thr Val Ser Gly Leu Ala Trp Thr Arg Gln
1 5 10 15

<210> 45
<211> 15
<212> PRT
<213> Human cytomegalovirus

<400> 45

Leu Thr Val Ser Gly Leu Ala Trp Thr Arg Gln Gln Asn Gln Trp
1 5 10 15

<210> 46
<211> 15
<212> PRT
<213> Human cytomegalovirus

<400> 46

Gly Leu Ala Trp Thr Arg Gln Gln Asn Gln Trp Lys Glu Pro Asp
1 5 10 15

<210> 47
<211> 15
<212> PRT
<213> Human cytomegalovirus

<400> 47

Thr Arg Gln Gln Asn Gln Trp Lys Glu Pro Asp Val Tyr Tyr Thr
1 5 10 15

<210> 48
<211> 15
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<213> Human cytomegalovirus

<400> 48

Asn Gln Trp Lys Glu Pro Asp Val Tyr Tyr Thr Ser Ala Phe Val
1 5 10 15

<210> 49
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<400> 49

Glu Pro Asp Val Tyr Tyr Thr Ser Ala Phe Val Phe Pro Thr Lys
1 5 10 15

<210> 50
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<400> 50

Tyr Tyr Thr Ser Ala Phe Val Phe Pro Thr Lys Asp Val Ala Leu
1 5 10 15

<210> 51
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<400> 51

Ala Phe Val Phe Pro Thr Lys Asp Val Ala Leu Arg His Val Val
1 5 10 15

<210> 52
<211> 15
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